Published in final edited form as:

Neuroscience. 2012 August 16; 217: 67–76. doi:10.1016/j.neuroscience.2012.04.053.

SK2 and SK3 Expression Differentially Affect Firing Frequency and Precision in Dopamine Neurons

Jason Deignan¹, Rafael Luján², Chris Bond¹, Arthur Riegel³, Masahiko Watanabe⁴, John T. Williams¹, James Maylie⁵, and John P. Adelman¹

¹Vollum Institute, Oregon Health & Science University, Portland, Oregon 97239, USA

²Department Ciencias Medicas, Instituto de Investigacion en Discapacidades Neurologicas (IDINE), Universidad de Castilla-La Mancha, Albacete, Spain, 02006

³Neurobiology of Addiction Center, University of South Carolina, Charleston, South Carolina 29425, USA

⁴Department of Anatomy, Hokkaido University School of Medicine, Sapporo 060-8638, Japan

⁵Department of Obstetrics and Gynecology, Oregon Health & Science University, Portland, Oregon 97239, USA

Abstract

The firing properties of dopamine (DA) neurons in the substantia nigra (SN) pars compacta are strongly influenced by the activity of apamin-sensitive small conductance Ca^{2+} -activated K^{+} (SK) channels. Of the three SK channel genes expressed in central neurons, only SK3 expression has been identified in DA neurons. The present findings show that SK2 was also expressed in DA neurons. Immuno-electron microscopy (iEM) showed that SK2 was primarily expressed in the distal dendrites, while SK3 was heavily expressed in the soma and, to a lesser extent, throughout the dendritic arbor. Electrophysiological recordings of the effects of the SK channel blocker apamin on DA neurons from wild type and SK^{-/-} mice show that SK2-containing channels contributed to the precision of action potential (AP) timing, while SK3-containing channels influenced AP frequency. The expression of SK2 in DA neurons may endow distinct signaling and subcellular localization to SK2-containing channels. Keywords: Substantia Nigra, Dopamine, SK channels, spontaneous activity, pacemaker

1. Introduction

1.1

The release of DA from midbrain dopaminergic neurons, such as SN neurons, influences voluntary movement, cognition, and motivational state (Bernheimer et al., 1973; Nieoullon, 2002). Perturbations in DA signaling are implicated in the pathologies of attention-deficit hyperactivity disorder, schizophrenia, and Parkinson's Disease (Bernheimer et al., 1973; Verhoeff, 1999; Goto and Grace, 2007; Tripp and Wickens, 2008). The consequences of DA signaling depend on the timing and amount of DA released, which reflect the different

© 2012 IBRO. Published by Elsevier Ltd. All rights reserved.

Corresponding Author: Dr. John P. Adelman, Vollum Institute, Oregon Health & Science University, 3181 SW Sam Jackson Park Rd., Portland, Oregon 97239, USA. adelman@ohsu.edu Tel: 1 (503) 494-5450 Fax: 1 (503) 494-4590.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

activity patterns exhibited by DA neurons (Gonon, 1988; Chergui et al., 1994; Fiorillo et al., 2008). *In vivo*, a tonic, low frequency firing of APs maintains basal DA levels in DA neuron projection areas. Brief transitions to a burst firing mode result in barrages of high frequency APs that transiently elevate DA levels to trigger DA mediated behaviors (Gonon and Buda, 1985; Chergui et al., 1994; Schultz, 2002). In freshly prepared brain slices with neurotransmission blocked, DA neurons exhibit an intrinsic, and regular pattern of AP firing, between 1–8 Hz (Grace and Onn, 1989; Lacey et al., 1989; Yung et al., 1991).

The Ca²⁺-dependent K⁺ channel, SK, contributes a sub-threshold outward current during pacemaking of SN neurons as well as the prominent afterhyperpolarization (AHP) following each AP that together regulates the regularity and frequency of AP firing (Shepard and Bunney, 1991; Nedergaard et al., 1993; Ping and Shepard, 1996). Selectively blocking SK channel activity with apamin increases the frequency and decreases the precision of AP firing, which can induce spontaneous transitions to burst firing (Shepard and Bunney, 1988; Shepard and Bunney, 1991; Ping and Shepard, 1996).

Previous studies have shown that primarily SK3 is expressed in SN DA neurons and its activity regulates the mean AP frequency (Wolfart et al., 2001). To further investigate the roles of SK3 channels in DA neurons, SK3 null mice were generated. The presence of an apamin sensitive current in the SK3 null mice suggested SK2 expression. In other neurons, SK2 channels are selectively targeted to dendrites where previous studies have concluded the DA neuron pacemaker resides, prompting us to further examine the roles of SK2 and SK3 using wild type and knockout mice. Using immuno-electron microscopy (iEM) and electrophysiology, the results from adult mice show that SK2 was expressed in DA neurons, and that SK2- and SK3-containing channels provided differential influences on DA neuron activity. This reflects the selective expression of SK2 in the dendrites, while SK3 is expressed in both the dendrites and the soma.

2. Materials and Methods

All experiments utilized C57/BL6 mice that were a minimum of 21 days old. The Institutional Animal Care and Use Committee approved all animal handling and protocols.

2.1 Generation of SK null mice

SK2^{-/-} mice—The generation of SK2 null mice has been previously described (Bond 04). **SK3^{-/-} mice**. A single lox P site was introduced into the first exon of the mouse SK3 gene, 200 bp 5' of the start of translation. The coding sequence for the neomycin resistance gene (neo), flanked by frt sites and followed by a single loxP site, was inserted into intron 1. Five chimeric mice derived from implantation of the same ES cell clone gave rise to germ line founders. Each of these lines have been crossed to an FLP-expressing mouse to remove the neo coding sequence, and subsequently backcrossed to C57Bl/6J for > 10 generations. Floxed mice were crossed to a global Cre expressing mouse (Schwenk et al., 1995) resulting in SK3^{-/-} mice. The removed protein sequence constitutes the intracellular N-terminus and the first transmembrane domain. Probing Western blots prepared using protein extracts from wild type and SK3^{-/-} mouse brains with anti-SK3 antibody (Alomone Labs) failed to detect a signal only from the null mice (not shown).

2.2 Real-time PCR

Total RNA from microdissections of 600 μm midbrain slices was isolated using Tri-reagent according to manufacturer's protocol. Total RNA was reverse-transcribed by MMLV reverse transcriptase (Invitrogen) in the presence of random hexamers but without dithiothreitol. Real-time PCRs were performed in triplicate for each SK transcript in each

genotype, and expression levels were determined by comparison to 18S rRNA. The amplicon for 18S was 76 bp (primers: CCGCAGCTAGGAATAATGGA, CCCTCTTAATCATGGCCTCA); for SK1, 118 bp (primers: GCTCTTTTGTCTTGAAATGCC, CAGTCGTCGGCACCATTGTCC); for SK2, 151 bp (primers: GTCGCTGTATTCTTTAGCTCTG, ACGCTCATAAGTCATGGC); for SK3, 148 bp (primers: GCTCTGATTTTGGGATGTTT, CGATGATCAAACCAAGCAGG ATGA). All SK amplicons span an intron. The efficiencies of the primer pairs were tested in a validation experiment using serial dilutions of a wild type cDNA (slope of ΔC_t (SK_{Ct}-18S_{Ct}) <0.1; not shown). C_t , the threshold cycle, indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold. The reaction master mix, consisting of 10X buffer, Mg (C_f = 4mM), dNTPs (C_f = 200 mM), Platinum taq polymerase (Invitrogen, Carlsbad, CA) (0.6 units/20 μ l reaction) and SYBR Green (Molecular Probes) (0.5X manufacturer's recommended concentration), was aliquoted, the cDNA substrates added, and then further aliquoted and primers added (C_f = 200 nM). Reactions were then split into triplicates for amplification in an MJ Research Opticon DNA Engine with cycling parameters 95°C, 2 minutes 1X; 95°C, 30 seconds/64°C, 45 seconds, with fluorescence read at 78°C for 40 cycles. A melting curve and gel electrophoresis analysis verified that a single product was amplified in all reactions. For each run, the relative mRNA level was determined by the expression $2^{-\Delta\Delta C_t}$ (ΔC_t (SK_{Ct}-18S_{Ct}) within each genotype, $\Delta\Delta C_t$ (ΔC_t _{SK transgene} - ΔC_t _{wildtype}) (ABO Prism 7700 Sequence Detection System, user bulletin 2). The mean and standard error of the value $2^{-\Delta\Delta C_t}$ for each SK mRNA in each genotype, across all runs, were plotted. Statistical significance was determined by 1-way ANOVA of ΔC_t values across all genotypes followed by Bonferroni t-test.

2.3 Immuno-electron microscopy

Mice were transcardially perfused with 0.1 M phosphate buffer (PB, pH 7.4) containing 4% paraformaldehyde, 0.05% glutaraldehyde and 15% picric acid. Brains were removed from the skull and 60 μ m thick sections were cut using a Vibratome. The sections were then processed for immunohistochemical detection of SK2 or SK3 and TH using double labelling pre-embedding techniques, as described previously (Koyrakh et al., 2005).

Antibodies—The primary antibodies used were: rabbit anti-SK2 polyclonal antibody (custom), rabbit anti-SK3 polyclonal antibody (Alomone Labs), and mouse anti-TH monoclonal antibody (Calbiochem). The characteristics and specificity of the antibodies anti-SK2 subunit have been described elsewhere (Cueni et al., 2008; Lin et al., 2008).

2.4 Electrophysiology

Electrophysiological recordings were made using 220 μ m, horizontal, midbrain slices from wild type, SK2^{-/-} (Bond et al., 2004) or SK3^{-/-} mice. Mice anesthetized with isoflurane were decapitated and the brains rapidly removed. Acute horizontal sections were made using a Leica VT1000 vibratome (Leica Microsystems) in an ice slurry of cutting solution composed of (in mM): 119 NaCl, 26 NaHCO₃, 2.5 KCl, 1 NaH₂PO₄, 1.3 MgCl₂, 2 CaCl₂, 25 dextrose, 3 ascorbate, and 1 pyruvate and equilibrated with 95% O₂/5% CO₂. Slices recovered at 34°C in cutting solution equilibrated with 95% O₂/5% CO₂ for at least 30 min until used for recording. Slices were then transferred to a heated recording chamber (33°C) and perfused (3 ml/min) with ACSF composed of (in mM): 119 NaCl, 26 NaHCO₃, 2.5 KCl, 1 NaH₂PO₄, 1.3 MgCl₂, 2 CaCl₂, 25 glucose, equilibrated with 95% O₂/5% CO₂. DA neurons were visualized with a CCD camera mounted on an Olympus BX-51 microscope equipped with a 60x, 0.9 N.A., water immersion objective and modified Dodt contrast enhancement optics. To minimize heterogeneity among DA neurons, cells from the medial SN were targeted for recording. DA neurons were identified by their location within the slice, spontaneous activity of 1 – 8 Hz, extracellular AP duration > 2 ms, and the presence of

I_h activated at a holding potential < -70 mV when in whole-cell mode (Grace and Bunney, 1983; Kita et al., 1986; Grace and Onn, 1989). Control ACSF was supplemented with the following antagonists to isolate intrinsic properties of DA neurons: SR 95531 (5 μ M; GABA_A), CGP55845 (2 μ M; GABA_B), Sulpiride (100 nM; D₂ receptors), D-AP5 (50 μ M; NMDA receptors), and CNQX (25 μ M; AMPA receptors).

Patch pipettes were pulled from borosilicate glass capillary tubing (Sutter, Novato CA), and had tip resistances between 1.5–3.5 M Ω . Whole-cell voltage clamp experiments were conducted with an internal solution containing (in mM): 115 KMeSO₄, 20 NaCl, 1.5 MgCl₂, 10 HEPES, 0.1 EGTA, 2 Mg-ATP, 0.2 Na⁺-GTP, and 10 Na-phosphocreatine and adjusted to pH 7.3 with KOH and 290 mOsm. Whole cell voltage clamp recordings were made using an Axopatch 200A amplifier (Molecular Devices, Sunnyvale, CA) and AxoGraph X software (AxoGraph, Sydney, Australia). Data were digitized at 10 KHz with a Digidata 1322A digitizer (Molecular Devices, Sunnyvale, CA) and filtered at 2 KHz.

Uncompensated, series resistances ranged from 5 to 20 M Ω . Series resistance was compensated at 75–80% and cells in which the series resistance changed by more than 20% were discarded. Extracellular recordings were made with ACSF filled pipettes. Cells exhibiting spontaneous activity were selected for recording. Recordings were made using either an Axopatch 200A or Multiclamp 700A amplifier (Axon Instruments). Records were digitized at 5 KHz and filtered at 2 KHz.

Analysis and Statistics—Data were analyzed with Igor Pro (Wavemetrics, Lake Oswego, OR) and statistics were calculated using the statistical software environment R (R Foundation for Statistical Computing). Mean ISI measurements were not significantly different than normal (Shapiro Wilks test $p > 0.1$). CV measurements were log-transformed to enhance symmetry and approximately equalize within-group variance. The transformed CV measurements and ISI measurements were analyzed using ANOVA, and significant differences identified with Fisher's least significant difference tests. P values < 0.05 were considered significant. ANOVA analyses of log-CV data identified the same significant effects as non-parametric tests of CV measurements, but were preferred because ANOVA provided an estimate of the effect magnitude. Data were presented as mean $\pm$ S.E.M.

3. Results

3.1 SK2 and SK3 are expressed in DA neurons

Whole cell voltage clamp recordings were performed on brain slices prepared from wild type, SK2^{-/-} and SK3^{-/-} mice. No differences in input resistance (M Ω) were detected between the genotypes (wild type 180 ± 17 , $n = 9$; SK2^{-/-} 242 ± 45 , $n = 5$; SK3^{-/-} 164 ± 45 ; $n = 5$). Voltage steps from -70 mV to 0 mV, for 50 ms, followed by repolarization to -50 mV elicited outward tail currents that were predominantly blocked by apamin (Figure 1A–C). SK channel-mediated currents (I_{SK}) were obtained by subtracting the residual current recorded following apamin (200 nM) application from that recorded under control conditions. Apamin sensitive currents, I_{SK} , were observed in all three genotypes and the decay of the I_{SK} currents were well described by single exponential functions. The peak of the I_{SK} from wild type DA neurons (1175 ± 117 pA, $n = 9$) was significantly larger than either the I_{SK} from SK2^{-/-} DA neurons (490 ± 132 pA; $n = 5$; $P < 0.01$) or the I_{SK} from SK3^{-/-} DA neurons (122 ± 23 pA; $n = 5$; $P < 0.001$; Figure 1D). No significant differences were found between the decay kinetics of the three groups (τ_{decay} (ms): wild type, 199 ± 16 , $n = 8$; SK2^{-/-}, 188 ± 22 , $n = 5$; SK3^{-/-}, 258 ± 71 ; $n = 5$).

Apamin-sensitive SK channel activity strongly influences the firing characteristics of DA neurons (Shepard and Bunney, 1988; Shepard and Bunney, 1991; Nedergaard et al., 1993; Ping and Shepard, 1996; Chan et al., 2007). Previous work showed that SK3 channel

activity regulates AP frequency in DA neurons, but did not find evidence of functional SK2 channel expression (Wolfart et al., 2001). Therefore the finding of apamin-sensitive currents in SK3^{-/-} mice might reflect compensatory expression of SK2. To determine whether SK2, as well as SK3 is expressed in wild type DA neurons iEM was performed. The specificity of the SK2 antibody has been previously demonstrated using sections from SK2^{-/-} mice (Lin et al., 2008). Similarly, the SK3 antibody did not stain sections prepared from SK3^{-/-} mice (not shown). DA neurons were identified by tyrosine-hydroxylase (TH) immunoreactivity (Figure 2A,B,D,E). In TH-positive neurons, SK2 immunoparticles were sparsely distributed and almost exclusively found in the dendritic plasma membrane (Figure 2A,B). For SK2 1.7% (2 immunoparticles) were in the soma, 26.3% (31 immunoparticles) were in thick dendrites (> 1μm²; presumably primary or secondary dendrites) and 72% (85 immunoparticles) were in thin dendrites (< 1μm²; presumably tertiary dendrites). In contrast, SK3 immunoparticles labelled both the somatic plasma membrane and the plasma membrane of dendrites (Figure 2D,E). For SK3, 29.2% (102 immunoparticles) were in the soma, 36.7% (128 immunoparticles) were in thick dendrites (> 1μm²), and 34.1% (119 immunoparticles) were in thin dendrites (< 1μm²). These results demonstrate that SK2 is expressed in wild type DA neurons of the SN in a pattern that partly overlaps the subcellular distribution of SK3.

3.2 SK channels influence both the rate and timing of action potentials

To investigate the roles of SK2 and SK3 in regulating intrinsic firing properties, loose cell-attached recordings were made from spontaneously active wild type DA neurons. DA neurons were identified by the presence of pacemaker firing, broad extracellular APs (> 2 ms), and sensitivity to bath application of DA following experiments. All experiments were performed in the presence of synaptic blockers to isolate the intrinsic properties of recorded cells (see Methods). After obtaining at least 15 min of stable control recording, the SK channel blocker, apamin (200 nM), was bath applied. In wild type neurons, apamin application decreased the interspike interval (ISI) (Fig. 3A). On average apamin decreased the ISI in 6 out of 7 of the recorded cells (control: 452 ± 38 ms; apamin: 325 ± 35 ms; $P < 0.01$, $n = 7$), corresponding to a 28 ± 13% decrease in the ISI. The leftward shift of the bottom portion of the cumulative probability histograms shows that apamin selectively increased the proportion of short intervals. (Figure 3C). The regularity of ISIs in wild type DA neurons was also affected by the blockade of SK channels as evidenced by the obvious change in regularity of AP firing in the representative traces and in the increased range of ISIs plotted versus time in Figure 3A. Histograms of the ISI (Fig. 3B) reveal that apamin application both decreases the mean and increases the standard deviation of ISIs as shown by the increased width of the histogram. To quantify the precision of firing the coefficient of variation (CV) was determined, which increased in 7 of 7 cells (control: 0.05 ± 0.01; apamin: 0.30 ± 0.16, $P < 0.01$) demonstrating decreased regularity of firing after apamin application (Figure 3D).

To determine the relative contribution of SK2 and SK3 to these firing properties recordings were made from SK^{-/-} mice. In slices prepared from SK2^{-/-} mice the mean ISI was significantly longer than that of wild type DA neurons (wild type: 452 ± 38 ms; SK2^{-/-}: 644 ± 76 ms; $P < 0.05$). In SK2^{-/-} mice, the effects of apamin can be attributed to the blockade of SK3-containing channels. As in wild type DA neurons, apamin application to SK2^{-/-} DA neurons decreased the ISI (Fig. 4A). On average apamin decreased the mean ISI by 22 ± 16% (control: 644 ± 76 ms; apamin: 492 ± 52 ms, $n = 9$; $P < 0.01$; Figure 4A). While apamin decreased the mean ISI in SK2^{-/-} DA neurons, it remained significantly greater than that of wild type DA neurons (wild type, apamin: 325 ± 35 ms; SK2^{-/-}, apamin: 492 ± 52 ms; $P < 0.05$). In the absence of SK2 expression, blocking SK3-containing channel activity decreased firing precision as shown by the increase of the width of the ISI histogram and

increase in CV (control: 0.08 ± 0.02 ; apamin: 0.34 ± 0.16 , $P < 0.05$; Figure 4B,D). Similar to wild type DA neurons, the cumulative probability histogram of ISIs revealed a selective effect on the proportion of shorter intervals. Apamin application significantly decreased 72% of ISIs ($P < 0.05$; Figure 4C).

Experiments were performed on slices prepared from SK3^{-/-} mice where the effects of apamin can be attributed to the blockade of SK2-containing channels. In SK3^{-/-} DA neurons, apamin did not affect the mean ISI (control: 490 ± 72 ms; apamin: 466 ± 84 ms, $n = 8$; $P = 0.38$; Figure 6). The decreased regularity of the ISIs in apamin is evident in both the example traces and ISI diary plot (Figure 5A). SK2 channel blockade selectively broadened the distribution of ISIs as indicated by the increase of the ISI histogram width and increased CV (control: 0.08 ± 0.01 ; apamin: 0.17 ± 0.04 ; $p = 0.01$; Figure 5B,D). The effects of apamin on mean ISI and CV are summarized the bar graph in Figure 6, which clearly shows that apamin decreases ISI only in WT and SK2^{-/-} mice whereas it decreases precision in both SK2^{-/-} and SK3^{-/-} mice.

The results presented above using 200 nM apamin to block SK channels in SK3^{-/-} DA neurons suggest that SK2 channels may selectively influence the regularity of action potentials while the results from SK2^{-/-} DA neurons suggest SK3 channels may selectively influence firing frequency. Homomeric SK2 channels are ~50-fold more sensitive to block by apamin compared to homomeric SK3 channels (EC_{50} ~100 pM vs ~5 nM; Lamy et al., 2010). Therefore, application of 300 pM apamin should block greater than 90% of SK2 channels while blocking less than 10% of SK3 channels. As shown in Fig 7, 300 pM apamin increased the CV (control: 0.04 ± 0.006 ; 300 pM apamin 0.07 ± 0.02 ; $p < 0.05$; $n=15$), resulting in a $54\% \pm 19\%$ increase in the CV, but did not alter firing frequency (control: 411 ± 41 ms; 300 pM apamin 419 ± 41 ms; $p > 0.05$; $n=15$). SK2 or SK3 induces compensatory changes that reduce Ca^{2+} influx during the voltage protocol, resulting in a smaller apamin sensitive tail current.

4. Discussion

4.1

The results presented here show that both SK2 and SK3 are expressed in DA neurons of the SN. The iEM results demonstrate that SK2 and SK3 have partially overlapping subcellular distributions within DA neurons. Electrophysiological recordings show that SK2 channels selectively contribute to AP timing while SK3 channels impact both AP frequency and timing.

The iEM clearly establishes that SK2 is expressed exclusively in the dendrites of DA neurons. Since we cannot unambiguously identify each dendrite with the parent soma this raises the question of whether SK2 is expressed in every DA neuron. However, apamin (200 nM) blocked tail currents in all SK3^{-/-} DA neurons tested, and applying a lower concentration of apamin (300 pM) that selectively blocks SK2 channels to wild type DA neurons decreased the precision of AP firing in 13 out of 15 DA neurons tested, suggesting that functional SK2-containing channels are expressed in most, if not all, DA neurons in the medial SN.

To gain insight into the distinct roles of SK2 and SK3, studies were performed on the respective null mice. In principle this should allow a clear delineation of the roles for SK2 and SK3, however, the mice show indications of compensatory alterations. This was first apparent from the tail current measurements, as the sum of apamin sensitive tail currents from SK2^{-/-} and SK3^{-/-} DA neurons is less than the apamin sensitive tail current in wild type DA neurons, despite a likely upregulation of SK3 expression in SK2^{-/-} mice as

suggested by qPCR for SK3 mRNA. This might, in part, reflect a more dendritic expression profile for the remaining SK channels in each of the null mice, that would minimize their contribution to somatically recorded currents (i.e. lack of dendritic space clamp). It is also possible that the absence of

To further characterize SK expression levels, qPCR was performed using RNA extracted from each genotype. Tissue samples containing the ventral tegmental area and SN were micro-dissected from 0.6 mm horizontal slices of mouse midbrain. The results showed that SK2 expression in SK3^{-/-} mice was not different from wild type ($P > 0.1$). In contrast, SK3 expression was increased to 142% in SK2^{-/-} mice compared to wild type ($P < 0.01$).

4.2

The effects on firing frequency (mean ISI) in the null mice clearly show that SK3 is required for apamin to affect the mean ISI; apamin reduced the mean ISI in wild type but did not affect the mean ISI in SK3^{-/-} DA neurons. This result corroborates previous conclusions that SK3 regulates AP frequency by contributing to the AHP (Wolfart et al., 2001). Moreover, cumulative probability distributions show that blocking SK3 (in wild type and SK2^{-/-} DA neurons) preferentially increases the proportion of short intervals, supporting the conclusion that, by contributing to AHP, SK3 acts as a low pass filter (Wolfart et al. 2001). The notable difference with previous studies is the remaining apamin sensitive component of the I-AHP in the absence of SK3. However, the dominant contribution of SK3 to the I-AHP and the lack of highly subtype selective antagonists preclude discriminating an SK2 contribution to the I-AHP. In addition, it is possible that the single cell PCR approach used by Wolfart, et al. efficiently extracted the somatic mRNA population but might have underestimated the SK2 mRNA content if the SK2 mRNA is localized to the dendrites where it undergoes local translation.

The results also indicate that there are compensatory changes in the SK3^{-/-} mice. For example, the mean ISIs for SK3^{-/-} and wild type DA neurons are not different, even though loss of SK3 would be expected to decrease the mean ISI to that of wild type DA neurons in the presence of apamin. There are also indications from measurements of the mean ISI that there have been compensatory changes in the SK2^{-/-} mice. The mean ISI of SK2^{-/-} DA neurons is larger than that for wild type. While this might reflect the upregulation of SK3, it is unlikely, as apamin application should normalize the mean ISI to that of wild type in apamin, but it does not. Apamin reduces the mean ISI in SK2^{-/-} DA neurons, but it remains larger than the mean ISI in wild type DA neurons in the presence of apamin.

The data suggest that both SK2 and SK3 channels affect the precision of AP firing (ISI-CV) as apamin reduced AP precision (increased the ISI-CV) in all genotypes, even though the ISI-CV values were not different in control conditions among the three genotypes. SK2 channels may selectively affect the precision of AP firing (ISI-CV) as apamin reduced AP precision (increased the ISI-CV) in wild type and SK3^{-/-} DA neurons. This is supported by the finding that apamin, while increasing the ISI-CV, did not affect the mean ISI in SK3^{-/-} DA neurons. The effect of apamin, increasing the ISI-CV in SK2^{-/-} DA neurons, suggests that SK3-containing channels also affect firing precision. However, this might reflect compensation in the SK2^{-/-} mice as indicated by the upregulation of SK3 mRNA.

4.3

Previous single cell PCR studies detected SK1 mRNA expression in DA neurons (Wolfart et al., 2001). While evidence for native protein expression of rat or mouse SK1 is lacking, heterologous expression studies have shown that rat or mouse SK1 subunits do not form functional homomeric channels in the plasma membrane. However, expression of chimeric

subunits containing the transmembrane core of rat SK1 did result in functional homomeric channels that were not blocked by apamin (D'Hoedt et al., 2004). Moreover, co-expression of mouse or rat SK1 with SK2 or SK3 (Benton et al., 2003; unpublished) did yield functional heteromeric channels that were apamin sensitive. Therefore, while we cannot rule out a contribution of SK1 subunits to the SK channels in SK2^{-/-} and SK3^{-/-} mice, the functional channels must contain the remaining apamin sensitive subunits.

Several findings using SK2^{-/-} and SK3^{-/-} mice support the conclusions that SK2-containing channels are functionally expressed in DA neurons and that they selectively influence the precision, but not the frequency, of AP firing. First, apamin blocked the tail currents in SK3^{-/-} DA neurons. Second, apamin decreased the precision of AP firing in SK3^{-/-} mice. Third, the effect of apamin on the precision of firing in SK3^{-/-} mice occurred despite the lack of apamin effect on AP frequency, also showing that SK2 does not compensate for the loss of SK3. Fourth, the deletion of SK2 affected both the mean ISI and tail current amplitudes. Nevertheless, there are clearly complications arising from compensatory changes in each of the null mice.

To directly test for the role of SK2 channels in DA neurons, a concentration of apamin (300 pM) was applied to wild type DA neurons that will selectively block >90% of SK2, but <10% of SK3 channels. The results support the conclusion that SK2 channels primarily influence the regularity of action potential firing, as the ISI CV increased while the frequency of action potentials was unaltered. Though unlikely, we cannot, however, formally exclude the possibility that the low dose of apamin is mediating its effects on the regularity of firing by blocking a small fraction (<10%) of SK3 channels that selectively contribute to action potential precision. Also, SK2 and SK3 subunits are capable of forming heteromeric channels in brain (Strassmaier et al., 2005), and heteromeric SK2/SK3 channels will have intermediate apamin sensitivity depending upon the precise subunit stoichiometry (Weatherall et al., 2011). Both SK2 and SK3 are present in the dendrites of DA neurons, therefore, we cannot rule out a contribution of dendritic SK3 subunit containing channels to the regularity of firing. Regardless, the selective modulation of CV by low doses of apamin support the conclusion that SK2 containing channels regulated action potential precision. Taken together with the effects of higher concentrations of apamin that also decreased the ISI, increased action potential frequency, these results suggest that SK2 and SK3 channels may serve complementary roles in regulating the activity of DA neurons.

SK2 and SK3 channels are similar in their Ca²⁺ gating properties (Köhler et al., 1996; Keen et al., 1999). SK2-containing channels in hippocampal CA1 neurons are subject to activity-dependent regulation and trafficking via protein kinase A phosphorylation of serine residues that are not conserved in the SK3 subunit sequence (Lin et al., 2008). This may endow SK2-containing channels in the dendrites of DA neurons with the ability to serve as selective targets for activity dependent changes and/or signal transduction mechanisms that affect AP precision.

Finally, the blockade of SK2 channels influences the precision of the pacemaker mechanism. Yet, the continued activity of DA neurons in apamin indicates that SK channels are not a necessary component for actual pacemaking. In contrast, Ca_v 1.3 (L-type) Ca²⁺ channel current is necessary for continued pacing (Putzier et al., 2009) and generates Ca²⁺ oscillations peaking in fine dendrites (Wilson and Callaway, 2000; Chan et al., 2007; Kuznetsova et al., 2010). In the presence of TTX, SK channels are activated by Ca_v 1.3 mediated Ca²⁺ influx (Nedergaard et al., 1993). This interaction may still occur during normal pacemaker activity, offering a possible mechanism by which apamin influences the ISI-CV. SK channels provide a strong hyperpolarizing influence following an action potential that de-inactivates voltage gated channels (Na_v and Ca_vs), standardizing the

number of channels available for the subsequent pacemaker potential and AP. Dendritic SK2 channels are positioned to fulfill this role for the Ca_v channels implicated as part of the pacemaker (Putzier et al., 2009; Kuznetsova et al., 2010).

Acknowledgments

This work was supported by grants from the NINDS (5F31NS064863) to JD, the NIH (NS038880 and NS065855) to JPA, and (MH081860) to JM, the Ministerio de Ciencia e Innovación (BFU-2006-01896) and Consolider-Ingenio (CSD2008-00005) and from Consejería de Educación y Ciencia, Junta de Comunidades de Castilla-La Mancha (PAI08-0174-6967) to R.L.

Abbreviations

AHP	afterhyperpolarization
AP	action potential
Ca_v	voltage dependent calcium channel
CV	coefficient of variation
DA	Dopamine
iEM	immuno-electron microscopy
ISI	inter-spike interval
ISI-CV	coefficient of variation of the inter-spike interval
I_{SK}	SK mediated current
Na_v	voltage dependent sodium channel
SK	$\text{K}_{\text{Ca} 2}$, small-conductance calcium-activated potassium channel
SN	substantia nigra
TH	tyrosine hydroxylase
TTX	tetrodotoxin (voltage dependent sodium channel antagonist)

References

- Benton DC, Monaghan AS, Hosseini R, Bahia PK, Haylett DG, Moss GW. Small conductance Ca^{2+} -activated K^{+} channels formed by the expression of rat SK1 and SK2 genes in HEK 293 cells. *The Journal of Physiology*. 2003; 553:13–19. [PubMed: 14555714]
- Bernheimer H, Birkmayer W, Hornykiewicz O, Jellinger K, Seitelberger F. Brain dopamine and the syndromes of Parkinson and Huntington. Clinical, morphological and neurochemical correlations. *J Neurol Sci*. 1973; 20:415–455. [PubMed: 4272516]
- Bond CT, Herson PS, Strassmaier T, Hammond R, Stackman R, Maylie J, Adelman JP. Small conductance Ca^{2+} -activated K^{+} channel knock-out mice reveal the identity of calcium-dependent afterhyperpolarization currents. *J Neurosci*. 2004; 24:5301–5306. [PubMed: 15190101]
- Chan CS, Guzman JN, Ilijic E, Mercer JN, Rick C, Tkatch T, Meredith GE, Surmeier DJ. ‘Rejuvenation’ protects neurons in mouse models of Parkinson’s disease. *Nature*. 2007; 447:1081–1086. [PubMed: 17558391]
- Chergui K, Suaud-Chagny MF, Gonon FG. Nonlinear relationship between impulse flow, dopamine release and dopamine elimination in the rat brain in vivo. *Neuroscience*. 1994; 62:641–645. [PubMed: 7870295]
- Cueni L, Canepari M, Lujan R, Emmenegger Y, Watanabe M, Bond CT, Franken P, Adelman JP, Lüthi A. T-type Ca^{2+} channels, SK2 channels and SERCAs gate sleep-related oscillations in thalamic dendrites. *Nat Neurosci*. 2008; 11:683–692. [PubMed: 18488023]

- D'Hoedt D, Hirzel K, Pedarzani P, Stocker M. Domain analysis of the calcium-activated potassium channel SK1 from rat brain. Functional expression and toxin sensitivity. *The Journal of biological chemistry*. 2004; 279:12088–12092. [PubMed: 14761961]
- Fiorillo CD, Newsome W, Schultz W. The temporal precision of reward prediction in dopamine neurons. *Nat Neurosci*. 2008
- Gonon FG. Nonlinear relationship between impulse flow and dopamine released by rat midbrain dopaminergic neurons as studied by in vivo electrochemistry. *Neuroscience*. 1988; 24:19–28. [PubMed: 3368048]
- Gonon FG, Buda MJ. Regulation of dopamine release by impulse flow and by autoreceptors as studied by in vivo voltammetry in the rat striatum. *Neuroscience*. 1985; 14:765–774. [PubMed: 2986044]
- Goto Y, Grace AA. The Dopamine System and the Pathophysiology of Schizophrenia: A Basic Science Perspective. *Int Rev Neurobiol*. 2007; 78C:41–68. [PubMed: 17349857]
- Grace AA, Bunney BS. Intracellular and extracellular electrophysiology of nigral dopaminergic neurons--I. Identification and characterization. *Neuroscience*. 1983; 10:301–315. [PubMed: 6633863]
- Grace AA, Onn SP. Morphology and electrophysiological properties of immunocytochemically identified rat dopamine neurons recorded in vitro. *J Neurosci*. 1989; 9:3463–3481. [PubMed: 2795134]
- Keen JE, Khawaled R, Farrens DL, Neelands T, Rivard A, Bond CT, Janowsky A, Fakler B, Adelman JP, Maylie J. Domains responsible for constitutive and Ca(2+)-dependent interactions between calmodulin and small conductance Ca(2+)-activated potassium channels. *J Neurosci*. 1999; 19:8830–8838. [PubMed: 10516302]
- Kita T, Kita H, Kitai ST. Electrical membrane properties of rat substantia nigra compacta neurons in an in vitro slice preparation. *Brain Res*. 1986; 372:21–30. [PubMed: 3708356]
- Köhler M, Hirschberg B, Bond CT, Kinzie JM, Marrion NV, Maylie J, Adelman JP. Small-conductance, calcium-activated potassium channels from mammalian brain. *Science*. 1996; 273:1709–1714. [PubMed: 8781233]
- Koyrakh L, Lujan R, Colón J, Karschin C, Kurachi Y, Karschin A, Wickman K. Molecular and cellular diversity of neuronal G-protein-gated potassium channels. *J Neurosci*. 2005; 25:11468–11478. [PubMed: 16339040]
- Kuznetsova AY, Huertas MA, Kuznetsov AS, Paladini CA, Canavier CC. Regulation of firing frequency in a computational model of a midbrain dopaminergic neuron. *Journal of computational neuroscience*. 2010; 28:389–403. [PubMed: 20217204]
- Lacey MG, Mercuri NB, North RA. Two cell types in rat substantia nigra zona compacta distinguished by membrane properties and the actions of dopamine and opioids. *J Neurosci*. 1989; 9:1233–1241. [PubMed: 2703874]
- Lamy C, Goodchild SJ, Weatherall KL, Jane DE, Liégeois JF, Seutin V, Marrion NV. Allsteric block of KCa2 channels by apamin. *J Biol Chem*. 2010; 285:27067–27077. [PubMed: 20562108]
- Lin MT, Lujan R, Watanabe M, Adelman JP, Maylie J. SK2 channel plasticity contributes to LTP at Schaffer collateral-CA1 synapses. *Nat Neurosci*. 2008; 11:170–177. [PubMed: 18204442]
- Nedergaard S, Flatman JA, Engberg I. Nifedipine- and omega-conotoxin-sensitive Ca²⁺ conductances in guinea-pig substantia nigra pars compacta neurones. *The Journal of Physiology*. 1993; 466:727–747. [PubMed: 8410714]
- Nieoullon A. Dopamine and the regulation of cognition and attention. *Prog Neurobiol*. 2002; 67:53–83. [PubMed: 12126656]
- Ping HX, Shepard PD. Apamin-sensitive Ca(2+)-activated K⁺ channels regulate pacemaker activity in nigral dopamine neurons. *Neuroreport*. 1996; 7:809–814. [PubMed: 8733751]
- Putzier I, Kullmann PHM, Horn JP, Levitan ES. Cav1.3 Channel Voltage Dependence, Not Ca²⁺ Selectivity, Drives Pacemaker Activity and Amplifies Bursts in Nigral Dopamine Neurons. 2009:1–6.
- Schultz W. Getting formal with dopamine and reward. *Neuron*. 2002; 36:241–263. [PubMed: 12383780]

- Schwenk F, Baron U, Rajewsky K. A cre-transgenic mouse strain for the ubiquitous deletion of loxP-flanked gene segments including deletion in germ cells. *Nucleic Acids Res.* 1995; 23:5080–5081. [PubMed: 8559668]
- Shepard PD, Bunney BS. Effects of apamin on the discharge properties of putative dopamine-containing neurons in vitro. *Brain Res.* 1988; 463:380–384. [PubMed: 3196925]
- Shepard PD, Bunney BS. Repetitive firing properties of putative dopamine-containing neurons in vitro: regulation by an apamin-sensitive Ca(2+)-activated K⁺ conductance. *Experimental brain research Experimentelle Hirnforschung Expérimentation cérébrale.* 1991; 86:141–150.
- Strassmaier T, Bond CT, Sailer CA, Knaus H-G, Maylie J, Adelman JP. A novel isoform of SK2 assembles with other SK subunits in mouse brain. *J Biol Chem.* 2005; 280:21231–21236. [PubMed: 15797870]
- Tripp G, Wickens JR. Research Review: Dopamine transfer deficit: a neurobiological theory of altered reinforcement mechanisms in ADHD. *J Child Psychol & Psychiat.* 2008; 49:691–704. [PubMed: 18081766]
- Verhoeff NP. Radiotracer imaging of dopaminergic transmission in neuropsychiatric disorders. *Psychopharmacology (Berl).* 1999; 147:217–249. [PubMed: 10639681]
- Wilson CJ, Callaway JC. Coupled oscillator model of the dopaminergic neuron of the substantia nigra. *Journal of Neurophysiology.* 2000; 83:3084–3100. [PubMed: 10805703]
- Weatherall KL, Seutin V, Liégeois JF, Marrion NV. Crucial role of a shared extracellular loop in apamin sensitivity and maintenance of pore shape of small-conductance calcium-activated potassium (SK) channels. *Proc Natl Acad Sci.* 2011; 108:18494–18499.
- Wolfart J, Neuhoﬀ H, Franz O, Roeper J. Differential expression of the small-conductance, calcium-activated potassium channel SK3 is critical for pacemaker control in dopaminergic midbrain neurons. *J Neurosci.* 2001; 21:3443–3456. [PubMed: 11331374]
- Yung WH, Häusser M, Jack JJB. Electrophysiology of dopaminergic and non-dopaminergic neurones of the guinea-pig substantia nigra pars compacta in vitro. *The Journal of Physiology.* 1991; 436:643–667. [PubMed: 2061849]

Highlights

- Dopamine neurons of the Substantia nigra express functional SK2 channels
- SK2 is predominantly expressed in fine dendrites ($< 1\mu\text{M}$) of nigral dopamine neurons
- SK2 blockade selectively affects the precision of the pacemaker mechanism
- SK3 expression is required for apamin to affect the frequency of spontaneous activity

Figure 1.

Apamin-sensitive currents recorded from wild type, SK2^{-/-} and SK3^{-/-} DA neurons. Pharmacological isolation of the apamin-sensitive SK current in wild type (A), SK2^{-/-} (B) and SK3^{-/-} (C) DA neurons. The currents were evoked by a 50 ms depolarizing pulse to 0 mV from -70 mV holding potential, followed by a return to -50 mV. The voltage protocol is schematically presented above the data trace in panel A. The black traces are currents recorded in control solution, while the red traces are currents recorded after apamin (200 nM) application. To obtain the subtracted, apamin sensitive current (inset), each record was zeroed to the mean, steady state current following repolarization to -50 mV. Each trace is the average of four sequential trials. (D) Averages $\pm$ S.E.M. for the apamin-sensitive currents for each genotype. Scale bars: current, 0.2 nA, time, 0.2 s.

Figure 2.

SK2 and SK3 are expressed in wild type DA neurons. Electron micrographs from SN showing immunoparticles for SK channel subunits and TH immunoreactivity as detected using double labeling immunogold-HRP. (A, B) Immunoparticles for SK2 protein (arrows) were found along the plasma membrane in dendrites (Den) of TH-positive neurons. (D) Immunoparticles for SK3 (arrows) were detected along the plasma membrane of the soma (Cyt, cytoplasm; N, nucleus) and (D) the dendrites (Den) of TH-positive neurons labeled with the HRP reaction product. (C,F) Summary graphs showing the sub-cellular distribution of SK2 and SK3 immunoparticles, respectively. Scale bars: 500 nm.

Figure 3.

SK channel activity regulates the timing and frequency of APs in wild type DA neurons. Panels A,B, C_i , and D_i are derived from the same representative wild type neuron. (A) Top traces show examples of spontaneous APs recorded in the loose patch configuration from a wild type DA neuron in control solution (black) and after apamin application (red). Below is a diary plot of the mean ISIs binned over 2 s for the same cell. Apamin was added at time 0. The shaded areas denote the control (black) and apamin (red) ISIs used for graphs and statistical comparisons. (B) Histogram of ISIs recorded in control solution (black) and after apamin application (red) for the indicated period in Panel A. (C) Cumulative probability histograms of ISIs from a representative cell (C_i) and for all wild type cells (C_{ii}) in control solution (black) after apamin application (red). The black and red points denote the mean

ISIs. (D_i) Diary plot of the effect of apamin (added at time 0) on the CV of ISIs. Each point represents the CV calculated from 30 s epochs of ISIs. Error bars are mean $\pm$ S.E.M.

Figure 4. Effects of SK channel activity in SK2^{-/-} DA neurons. Panels A, B, C_i, and D_i are derived from the same representative SK2^{-/-} neuron. (A) Top traces show examples of spontaneous APs recorded in the loose patch configuration from an SK2^{-/-} DA neuron in control solution (black) and after apamin application (red). Below is a diary plot of the ISIs for the same cell. Apamin was added at time 0. The shaded areas denote the control (black) and apamin (red) ISIs used for graphs and statistical comparisons. (B) Histogram of ISIs recorded in control solution (black) and after apamin application (red). (C) Cumulative probability histograms of ISIs from panel A (C_i) and for all SK2^{-/-} DA neurons (C_{ii}) in control solution (black) after apamin application (red). The black and red points denote the mean ISIs. (D) Diary plot

of the effect of apamin (added at time 0) on the CV of ISIs. Each point represents the CV calculated from 30 s epochs of ISIs. Error bars are mean $\pm$ S.E.M.

Figure 5.

SK2-containing channels influence AP timing in SK3^{-/-} DA neurons. Panels A,B,Ci, and Di are derived from the same representative SK3^{-/-} neuron. (A) Top traces show example traces of spontaneous APs recorded in the loose patch configuration from an SK3^{-/-} DA neuron in control solution (black) and after apamin application (red). Below is a diary plot of the ISIs for the same cell. Apamin was added at time 0. The shaded areas denote the control (black) and apamin (red) ISIs used for graphs and statistical comparisons. (B) Histogram of ISIs recorded in control solution (black) and after apamin application (red). (C) Cumulative probability histograms of ISIs from panel B (C_i), and for all SK3^{-/-} DA neurons (C_{ii}) shown in control solution (black) after apamin application (red). The black and red points denote the mean ISIs. (D) Diary plot of the effect of apamin (added at time 0) on

the CV of ISIs. Each point represents the CV calculated from 30 s epochs of ISIs. Error bars are mean $\pm$ S.E.M.

Figure 6. Summary bar graphs showing the effects of apamin (200 nM) on the mean ISI (top) and ISI-CV (bottom) in the different genotypes examined.

Figure 7.

AP timing is selectively affected by blocking SK2-containing channels. The effects of 300 pM apamin on wild type DA neurons are shown for individual cells (grey) and population means $\pm$ S.E.M. (black). (A,B) Effects of apamin on CV and ISI, respectively. (C) The fold change relative to control for CV and ISI.